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14. ABSTRACT The long-term objective of this proposal is to identify mechanisms of prion transport to secondary lymphoreticular system (LRS) tissues. The hypothesis to be tested is that following peripheral exposure to prions; host proteins (e.g. complement) bind prions allowing for trapping by cells in the spleen and enhancing uptake by macrophages, which are cells that are responsible for destruction of foreign proteins. To investigate this hypothesis we will examine the disease development of a prion strain (DY TME) that does not replicate in the spleen of hamsters. We will use this system to provide details into the host factor(s) involved in transport of prions to cells in the LRS, such as spleen. We have shown differences in the susceptibility of HY and DY TME to phagocytosis and degradation by primary adherent peritoneal cells. We have shown differences in the spatial and temporal spread of the HY and DY TME agent in LRS tissues following intraperitoneal inoculation. We are currently investigating what cell types associate with these agents following inoculation and the proportion of each agent that is degraded.					
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Introduction

The long-term objective of this proposal is to identify mechanisms of prion transport to secondary lymphoreticular system (LRS) tissues. The hypothesis to be tested is that following peripheral exposure to prions, host proteins (e.g. complement) bind prions allowing for trapping by cells in the spleen and enhancing uptake by macrophages, which are cells that are responsible for destruction of foreign proteins. To investigate this hypothesis two animal models will be used. Genetically engineered mice that lack components of the complement system will be used to test the hypothesis that complement binding to PrP^{Sc} is involved in targeting of prions to cells in the spleen and uptake by macrophages. A second system will examine disease development of a prion strain (DY TME) that does not replicate in the spleen of hamsters. We will use this system to test the hypothesis that DY TME is not bound by complement resulting in its absence in the spleen. The mouse and hamster systems investigate prion interactions with complement components based on differences of host and strain properties, respectively. This study will provide details into the host factor(s) involved in transport of prions to cells in the LRS, such as spleen.

Body

Tissue distribution of HY & DY TME at early time points post-infection. As previously reported, we began to investigate the distribution of infectivity and PrP^{Sc} in hamsters infected with the HY and DY TME agent at early time points post-infection as outlined in task 4. In the preliminary experiment, hamsters were intraperitoneally inoculated with uninfected homogenate or 10^{4.5} LD₅₀ of DY TME or 10^{7.5} LD₅₀ of HY TME. At 7.5 hours, 2.5, 5, 10, 20 and 40 days post-infection, three animals for each inoculation group were sacrificed and peritoneal cells, spleen, mesenteric lymph node, medial iliac lymph node and submandibular lymph node were collected. We used detergent extraction and ultracentrifugation to enrich for PrP^{Sc} prior to Western blot analysis as previously described (Bartz et al., 2004). We found these time points and inoculum concentrations to be sub-optimal, and repeated the experiment using 10^{5.5} LD₅₀ of DY TME or 10^{8.5} LD₅₀ of HY TME. At 1, 2, 4, 8, 16, and 32 hours post-infection, three animals for each inoculation group were sacrificed and peritoneal cells, spleen, mesenteric lymph node, medial iliac lymph node, and submandibular lymph node were collected. We used detergent extraction and ultracentrifugation to enrich for PrP^{Sc} prior to Western blot analysis. Our results indicate differences between the two TME strains (Table 1). HY PrP^{Sc} is present in the peritoneal cells, medial iliac lymph

Table 1. Distribution of PrP ^{Sc} following intraperitoneal inoculation with either the HY or DY TME agent												
	1h		2h		4h		8h		16h		32h	
	HY	DY	HY	DY	HY	DY	HY	DY	HY	DY	HY	DY
Peritoneal Cells	9/9 ^a	8/9	7/9	9/9	8/9	9/9	6/6	5/6	5/6	2/6	5/6	2/6
Medial Iliac Lymph Node	5/9	0/9	5/6	3/6	4/9	2/6	2/3	1/3	3/3	1/3	3/3	2/3
Mesenteric Lymph Node	0/6	0/9	2/6	0/6	2/9	0/9	1/3	0/3	1/3	0/3	1/3	0/3
Spleen	3/9	4/9	3/9	4/9	8/9	4/9	2/3	0/3	0/3	0/3	1/3	0/3

^a number of PrP^{Sc} positive animals / number of inoculated animals

node, and spleen as early as 1 hour post-infection, and remains in these tissues through 32 hours post-infection. HY PrP^{Sc} is present in the mesenteric lymph node by 2 hours post-infection, and remains in this tissue through 32 hours post-infection (Figure 1, Panel A). In contrast, DY PrP^{Sc} is present in the peritoneal cells and medial iliac lymph node as early as 1 hour post-infection, but is nearly eliminated by 32 hours post-infection. DY PrP^{Sc} is undetectable in the mesenteric lymph node at all time points, and is detectable in the spleen between

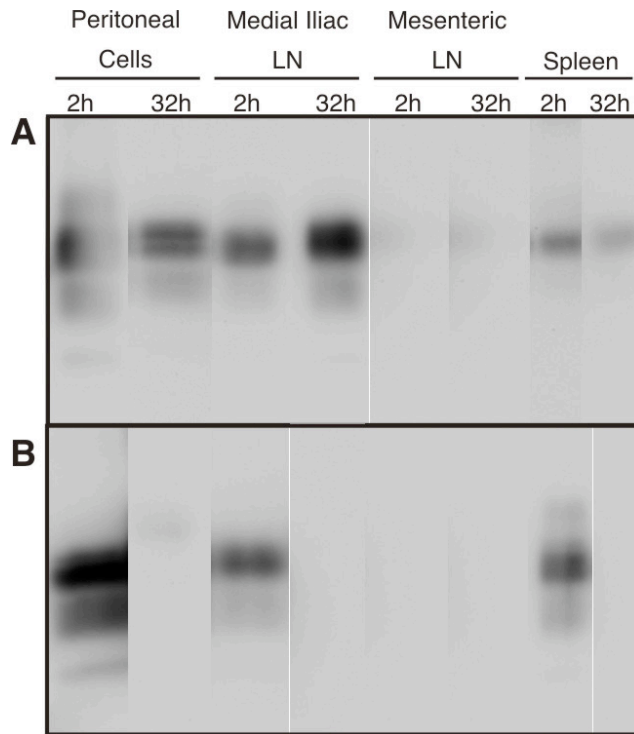


Figure 1. Strain specific reduction in PrP^{Sc} levels from secondary lymphoreticular system tissues. Western blot analysis of HY (panel A) and DY (panel B) PrP^{Sc} at select time points (2 hours and 32 hours) post-infection. HY PrP^{Sc} is present in the peritoneal cells (lanes 1 & 2), medial iliac lymph node (lanes 3 & 4), mesenteric lymph node (lanes 5 and 6) and spleen (lines 7 & 8) at 2 and 32 hours post-infection. DY PrP^{Sc} is present in the peritoneal cells, medial iliac lymph node and spleen at 2 hours post-infection (lanes 1, 3, & 7), but is nearly eliminated by 32 hours post-infection (lanes 2, 4, & 8). DY PrP^{Sc} is undetectable in the mesenteric lymph node at 2 and 32 hours post-infection (lanes 5 & 6).

1 and 4 hours post-infection, but is not detectable at 8, 16, and 32 hours post-infection (Figure 1, Panel B). We are currently examining tissue collected at 64 hours, 1, 2, and 4 weeks post-infection to determine PrP^{Sc} concentrations at these later time points.

To determine the localization of PrP^{Sc} in secondary lymphoreticular tissues, we are currently using immunohistochemical analysis to observe the specific cell types that associate with PrP^{Sc} post-inoculation. In this study, hamsters were intraperitoneally inoculated with uninfected homogenate or $10^{5.5}$ LD₅₀ of DY TME or $10^{8.5}$ LD₅₀ of HY TME. At 2, 4, 8, 16, 32, 64, and 128 hours post-infection, three animals for each inoculation group were sacrificed and perfused with PLP fixative as previously described (Bartz et al., 2003). Following fixation, the spleen, medial iliac lymph node, mesenteric lymph node, and submandibular lymph node were collected. We are currently performing PrP^{Sc} IHC analysis using the prion protein specific antibody, 3F4, as previously described (Bartz et al., 2003).

Macrophage degradation of TME PrP^{Sc}. The interactions of HY and DY TME PrP^{Sc} and macrophages are being investigated as outlined in task 6. Significant progress has been made co-culturing HY and DY TME with primary hamster adherent peritoneal cells. In these experiments, primary adherent peritoneal cells were collected via peritoneal lavage using 20 ml RPMI (Mediatech, Herndon, VA). The cells were counted using a hemocytometer (Hausser Scientific, Horsham, PA) and the cell concentration was adjusted to 10^6 cells per ml using pre-warmed (37°C) RPMI. A total of 10^5 cells per well were placed in a plastic 96-well cell culture plate. The cells were allowed to adhere to the plate for 2 hours prior to a series of washes with RPMI Media containing 5% fetal bovine serum (ATCC, Manassas, VA), 1mM L-glutamine (Gibco, Carlsbad, CA) and 100 U/ml of penicillin and 100 µg/ml of streptomycin (Sigma, St. Louis, MO). Adherent peritoneal cells are most likely macrophages based on their ability to phagocytose (data not shown).

To assess if hamster primary adherent peritoneal cells could degrade HY or DY PrP^{Sc}, PrP^{Sc} was incubated with either 10^5 hamster primary adherent cells or an equal volume of RPMI Media without cells as a control for non-peritoneal cell mediated PrP^{Sc} degradation. HY and DY TME brain homogenates were prepared in PBS and were not digested with proteinase K. 100 µg equivalents of brain homogenate in RPMI Media was added to each well. Samples in triplicate were collected at 1, 24, 48, 72, and 128 hours post-TME-infection. At each collection point the media was removed and saved. An equal volume of RPMI Media was added to the well to collect the remaining free PrP^{Sc} and was added to the previously collected media. The PrP^{Sc} in the media and wash is referred to as the media associated PrP. To the cells, 100µl of 0.1% w/v NLS was added to the well to dislodge the cells from the well and collected. The wells were then washed with 100 µl of 0.1% w/v NLS that was added to the first cell collection. The PrP^{Sc} collected in 0.1% NLS is referred to as cell-associated PrP. The levels of PrP in the media and associated with the cells were quantified using Western blot analysis with the anti-PrP monoclonal antibody, 3F4.

Using this system, we have identified strain-specific differences in the ability of primary hamster peritoneal cells to degrade PrP^{Sc}. In HY TME-infected peritoneal cells there was a reduction in PrP^{Sc} levels in the media through 72 hours post-infection at which point the media was changed to continue cell

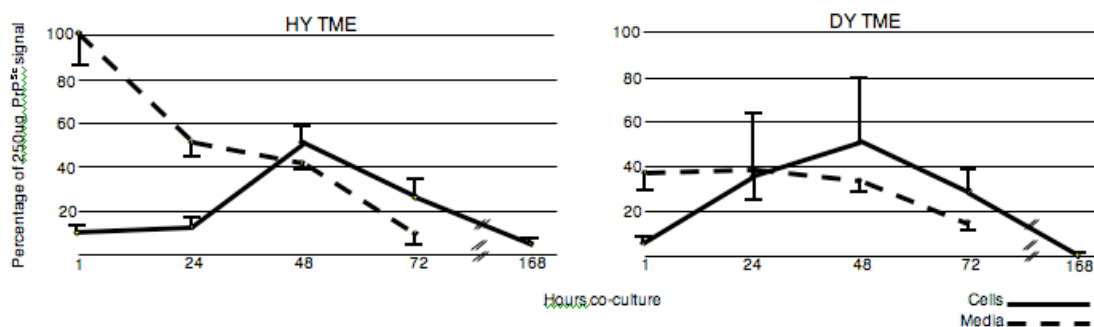


Figure 2. Strain specific reduction of PrP^{Sc} abundance in hamster macrophages. Abundance of HY or DY PrP^{Sc} (ug/ml) in the media (dashed line) or associated with primary hamster peritoneal cells (solid line) at selected time points (1, 24, 48, 72, & 168 hours) post co-culture.

growth (Figure 2). In macrophages infected with HY TME brain homogenate, the cell associated PrP^{Sc} level was detectable at low levels at 1 hour post-infection, the PrP^{Sc} levels increased at 24 and 48 hours post-infection and was still detectable at 168 hours post-infection (Figure 2). In DY TME infected macrophages there was no significant reduction in the levels of PrP^{Sc} in the media until 72 hours post-infection (Figure 2). In macrophages infected with DY TME-infected homogenate, cell-associated PrP^{Sc} was first detected at 1 hour post-infection and PrP^{Sc} levels increased until 48 hours when levels were reduced and were undetectable by 72 hours post-infection (Figure 2).

To summarize the differences between HY and DY TME-infected hamster peritoneal cells, HY PrP^{Sc} is detected in the peritoneal cells for a longer period of time than DY TME. This suggests that HY TME may be more resistant to degradation, and would not be cleared from the host unlike DY TME. In addition, the lower levels of HY PrP^{Sc} in the media when compared to DY PrP^{Sc} suggests that HY TME is more efficiently phagocytosed than DY TME. These data suggest that the macrophage may play a role in shuttling PrP^{Sc} from the periphery to secondary lymphoreticular tissues like spleen and lymph nodes.

Susceptibility of DY PrP^{Sc} to protease digestion. It is possible that the observed decrease in DY PrP^{Sc} in secondary lymphoreticular tissues is not due to an entire degradation of the prion protein, but due to a partial degradation of this protein that removes the 3F4 epitope. It has been shown that DY PrP^{Sc} is more susceptible to protease digestion than HY PrP^{Sc} (Bessen and Marsh, 1992). To determine the proportion of the DY PrP^{Sc} molecule that is degraded in these tissues, a series of prion protein specific antibodies will be used. Each of these antibodies, 8B4 (a gift from Man Sun SY), 3F4, and POM19 (a gift from A. Aguzzi) is specific to a different epitope along the prion protein. 8B4 is specific to amino acids 34-41, 3F4 is specific to amino acids 109-112, and POM19 is specific to amino acids 215-218. For these experiments, 250 µg equivalents of

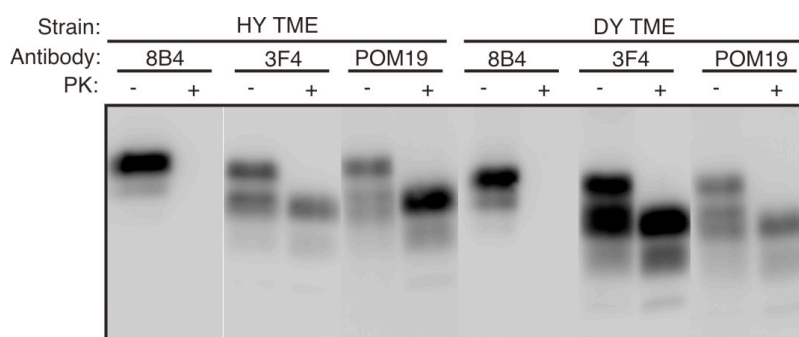


Figure 3. Western blot analysis of HY and DY TME using the 8B4, 3F4, and POM19 antibodies. Prior to PK digestion, the N-terminal portion of the protein is present in both TME strains (lanes 1 & 7), but following PK digestion, this portion is degraded (lanes 2 & 8). Following PK digestion, the 3F4 and POM19 epitopes remain intact for both TME strains (lanes 3-6 & 9-12) with DY PrP^{Sc} migrating 1-2 kDa faster than HY PrP^{Sc}.

uninfected, HY or DY TME-infected 10% w/v brain homogenates were incubated with 4 U/ml proteanase K (Roche Diagnostics, Mannheim, Germany) and size fractioned on 12.5% SDS-PAGE and transferred to Immobilon P as previously described (Bartz et al., 2004). The membrane was incubated with 8B4,

3F4, or POM19 in TTBS containing 5% Blotto (Bio-Rad Laboratories, Hercules, CA) overnight at 4°C. The membrane was washed with TTBS and incubated with goat anti-mouse antibody horseradish peroxidase conjugate (Pierce, Rockford, IL). The Western blot was developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce, Rockford, IL) according to manufactures instructions. The chemiluminescence signal was detected using a Kodak 2000R imaging station (Kodak, Rochester, NY). Following proteanase K digestion, the N-terminal portion of the prion protein is degraded. The epitope for 8B4 is degraded in both HY and DY TME strains, but the epitopes for 3F4 and POM19 remain after proteanase K digestion (Figure 3).

To determine the proportion of the HY and DY TME protein that is degraded in hamsters intraperitoneally inoculated with these agents, we will inoculate hamsters intraperitoneally with $10^{5.5}$ LD₅₀ of DY TME or $10^{8.5}$ LD₅₀ of HY TME. At 1, 2, 4, 8, 16, 32, and 64 hours post-infection, three animals for each inoculation group and each antibody, 9 animals total, will be sacrificed. The peritoneal cells, spleen, medial iliac lymph node, mesenteric lymph node, and submandibular lymph node will be collected. We will then enrich for PrP^{Sc} using detergent extraction and ultracentrifugation prior to Western blot analysis as previously described (Bartz et al., 2004). This data would help to determine what portion of the DY PrP^{Sc} protein is being degraded in the secondary lymphoreticular tissues.

Serum amyloid protein and the response of female hamsters to DY TME-infection. It is possible that the observed increase in incubation period in complement deficient animals is not due to a direct interaction of complement components with PrP^{Sc} but via an intermediate molecule. A possible candidate molecule is serum amyloid protein (SAP) that has been shown to bind amyloid and can also directly bind to C1q (Coe and Ross, 1990; Nauta et al., 2003). The hamster homolog of SAP is female protein, which is regulated by estrogen resulting in approximately 100-fold higher serum level of female protein compared to male hamsters (Coe & Ross, 1990). To investigate if this higher level of serum protein had an effect on DY TME incubation period and DY PrP^{Sc} properties, male and female hamsters were intracerebrally inoculated with $10^{3.8}$ LD₅₀ of DY TME. The hamsters were assessed daily for the onset of clinical symptoms of DY TME which is characterized by a progressive lethargy. Male hamsters had an incubation period of 169 ± 3 days (\pm SEM) while the female hamsters had an incubation period of 174 ± 4 days. The incubation period of DY TME in male and female hamsters was not statistically significant ($p > 0.05$ Student's T-test). This differs from previous studies that indicated in an eight percent reduction in the incubation period of hamsters i.c. inoculated with the 263K strain of hamster-adapted scrapie (Kimberlin & Walker, 1977) but is consistent with studies in mice where the incubation period in females is not uniformly shorter than in males (Outram, 1976).

Following intraperitoneal inoculation of male hamsters with the DY TME agent derived from males, the animals do not express clinical symptoms and DY PrP^{Sc} and the DY TME agent is undetectable in secondary lymphoreticular

Gender:	Male		Female				
Strain:	HY	DY	DY				
Tissue:	Br	Br	Br	MILN	MLN	SLN	Spl
Mg eq:	0.25	0.25	0.25	5.6	19.1	99.8	187.2

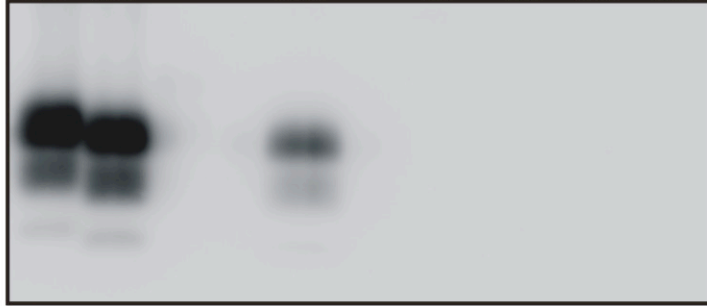


Figure 4. Western blot analysis of brain and secondary lymphoreticular system tissues from a female hamster intraperitoneally inoculated with DY TME. (Br-brain, MILN-medial iliac lymph node, MLN-mesenteric lymph node, SLN-submandibular lymph node, Spl-spleen).

tissues and brain (Bartz et al., 2005). To investigate if higher levels of serum protein had an effect on the ability of DY TME to cause disease following intraperitoneal inoculation, additional experimentation was necessary. $10^{4.5}$ LD₅₀ of DY TME from male hamsters was intraperitoneally

inoculated into female recipient hamsters. One out of the four female hamsters that was intraperitoneally inoculated exhibited DY TME clinical symptoms at 224 days post-inoculation. DY PrP^{Sc} was present in the brain at the time of sacrifice (Figure 4, lane 3). This is the first known case of DY TME causing disease following a non-neuronal route of inoculation. Following enrichment for PrP^{Sc} by detergent extraction and ultracentrifugation, none of the secondary lymphoreticular tissues tested contained detectable levels of DY PrP^{Sc} (Figure 4, lanes 4-7). It is possible that the DY TME agent was transiently located within these tissues, but was cleared by the time of clinical onset. To further study this possibility, we will repeat this experiment and collect secondary lymphoreticular tissues at early time points including 1, 2, 4, 8, 16, 32, 64, and 128 hours post-infection as described in section 1.

Key Research Accomplishments

1. Peritoneal cells and lymphoreticular system tissues from uninfected, HY TME and DY TME-infected hamsters have been collected and the spatial and temporal spread of PrP^{Sc} in these tissues has been determined by Western blot.
2. Secondary lymphoreticular system tissues from uninfected, HY TME and DY TME-infected hamsters has been collected and we are currently using immunohistochemistry to determine the cell types PrP^{Sc} is associated with.
3. Determined that female hamsters are susceptible to intraperitoneal DY TME infection unlike male hamsters.

4. Co-culture experiments of primary hamster peritoneal cells and PrP^{Sc} from HY and DY TME-infected hamsters demonstrated a strain specific response of PrP^{Sc} degradation.

Reportable Outcomes

None

Conclusions

We have initiated studies on the tissue distribution of HY and DY TME at early time points post-intraperitoneal infection. We have collected peritoneal cells and lymphoreticular system tissues and have analyzed these tissues for the presence of PrP^{Sc} through 32 hours post-infection. We are currently analyzing tissue that was collected between 64 hours and 4 weeks post-infection for the presence of PrP^{Sc}. We are also in the process of localizing the PrP^{Sc} in these tissues using immunohistochemical analysis. We have evidence of strain-specific phagocytosis and degradation of PrP^{Sc} by primary hamster peritoneal cells. We will begin to investigate the proportion of DY PrP^{Sc} that being degraded in secondary lymphoreticular tissues using a series of prion specific antibodies. Finally, we have demonstrated that female hamsters, that express higher levels of female protein than male hamsters, are susceptible to intraperitoneal inoculation with DY TME unlike male hamsters intraperitoneally inoculated with the same dose of DY TME.

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Appendices

None